A Fast and Sensitive LC/MS/MS Method for the Quantitation and Confirmation of 30 Benzodiazepines and Nonbenzodiazepine Hypnotics in Forensic Urine Samples

Cliquid™ Drug Screen & Quant Software for Routine Forensic Toxicology

3200 QTRAP® LC/MS/MS System

Overview

A fast and sensitive LC/MS/MS method for the detection of 27 benzodiazepines and 3 nonbenzodiazepine hypnotics in forensic urine samples was developed. Quantitation and confirmation of all compounds is possible at least one order of magnitude lower than the cut-off level of 100ng/mL.

The developed method is preconfigured in Cliquid™ Drug Screen & Quant Software which enables the use of this method in forensic toxicology or clinical research with automatic reporting of quantitative results and MRM ratio confirmation.

Introduction

Benzodiazepines and other non-benzodiazepine hypnotics, such as Zaleplon, Zolpidem, and Zopiclone are widely prescribed psychoactive drugs for the treatment of anxiety and sleep disorders. Frequently these substances lead to dependence and abuse and some of them can affect judgment and behavior. Thus these compounds are of great interest in forensic, toxicological and clinical research laboratories.

The screening for benzodiazepines with immunoassay tests does not provide enough sensitivity and specificity. The analysis using Gas Chromatography (GC) with different detectors is difficult or impossible because of thermal instability and requires time consuming derivatization and clean-up steps. Finally Liquid Chromatography (LC) with UV detection can not detect benzodiazepines at required concentration levels and lacks in selectivity.

LC coupled to Tandem Mass Spectrometric detection (MS/MS) with Electrospray Ionization (ESI) is the ideal technology for the analysis of polar and thermally labile drugs and their metabolites with highest sensitivity and specificity after a fast and simple sample preparation step. The developed LC/MS/MS method detects 30 analytes in a single chromatographic run using two Multiple Reaction Monitoring (MRM) transitions

to allow quantitation and confirmation. The detection of such a high number of transitions without sensitivity loss is possible due to the Linear Accelerator (LINAC®) collision cell of the 3200 QTRAP® LC/MS/MS System.

Experimental

Sample Preparation

100µL of internal standard solution (1µg/mL D_5-Diazepam and D_3-Doxepin) were added to 100µL of urine sample. After shaking 800µL water (LC grade) were added. The diluted sample was centrifuged and directly injected into the LC/MS/MS system.

Alternatively, urine samples can be hydrolyzed using ß-glucuronidase prior to analysis to detect benzodiazepine metabolites together with the precursor drugs.[1,2,3]

Liquid Chromatography

LC separation was carried out using a Shimadzu Prominence LC system equipped with 2 pumps, semi-micro gradient mixer, column oven and autosampler. An analytical Allure® PFP propyl column (Restek, Bellefonte, PA) 50x2.1mm with 5µm particles and 10x2.1 guard column was used with a mobile phase of (A) water with 0.2% formic acid and 2mM ammonium formate and (B) acetonitrile with 0.2% formic acid and 2mM ammonium formate. The gradient conditions are described in Table 1. The LC column was used at a temperature of 40°C and a volume of 30µL was injected.

Mass Spectrometry

MS/MS detection was carried out on a 3200 QTRAP® LC/MS/MS system equipped with a Turbo V™ source with Electrospray Ionization probe. The detected MRM transitions with corresponding compound depending parameters are given in Table 2 (Declustering Potential; DP and Collision Energy; CE). The first MRM was used to quantify the analyte while the ratio to the second MRM was used for confirmation. All MRM transitions were detected with a dwell time of 25ms and a pause time of 5ms.

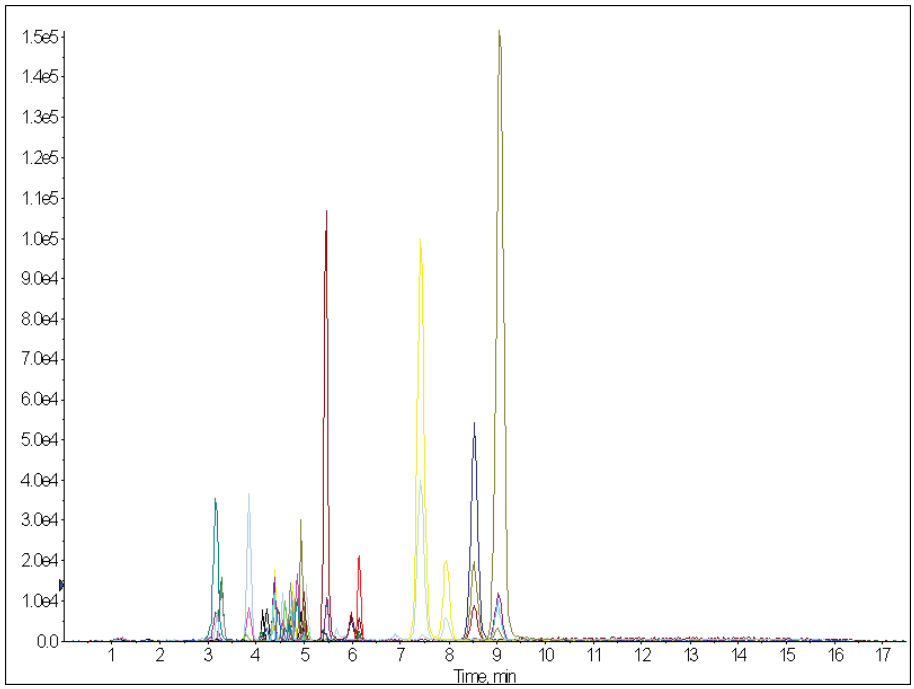


Figure 1. Chromatogram of a mix of drugs of 30 benzodiazepines and nonbenzodiazepine hypnotics spiked into a urine sample and analyzed by LC/MS/MS.

Cliquid™ Drug Screen & Quant Software

The developed method is preconfigured in Cliquid™ Drug Screen & Quant Software.

Cliquid Software provides a simple, four step workflow using preconfigured tests and report styles to make the operation of LC/MS/MS technology for routine forensic and toxicological testing easier. All presented results of forensic case samples are generated automatically and instantly after acquisition.

Results

An example chromatogram of all 30 benzodiazepines and nonbenzodiazepine hypnotics spiked into urine and analyzed by LC/MS/MS after sample preparation is shown in Figure 1. A list

TABLE 1. LC GRADIENT WITH (A) WATER + 0.2% FORMIC ACID + 2mM AMMONIUM FORMATE AND (B) ACETONITRILE + 0.2% FORMIC ACID + 2mM AMMONIUM FORMATE

Time (min)	Flow (mL/min)	% A	% B
0	0.5	90	10
10	1.0	10	90
15	1.0	10	90
15.5	0.5	90	10
17.5	0.5	90	10

of all retention times of all substances can be found in Table 1. The superior sensitivity of the method is highlighted by extracted MRM chromatograms presented in Figure 2. Signal-to-Noise varies depending on the ionization and fragmentation efficiency of each analyte. Thus Limits of Quantitation (LOQ) are different. However, all targeted compounds can be quantified in urine samples after a dilution step (10x) at a concentration of at least 10ng/mL. All LOQ values are listed in Table 2. The Coefficients of Variation (% CV) of all compounds below 10% indicate that the developed method allows reproducible quantitation even at low concentration levels.

The high selectivity of MRM detection allows compound specific detection without interference of urine matrix or other xenobiotics being present in the sample. Possible ion suppression effects during ESI caused by co-eluting matrix components were investigated using pooled urine. Figure 3 and 4 show that matrix effects depend on retention times and that they can be minimized or completely eliminated by dilution of the urine sample. For example, the signal of 7-Aminoclonazepam (t_R=3.3min) had strong matrix interference requiring dilution by a factor of 10 to minimize the ion suppression to less than 20% while the signal of Zolpidem (t_R=7.3min) was not effected by matrix at all.

Based on these studies a dilution factor of 10 was chosen to minimize matrix effects to a level of less than 20% for all analytes.

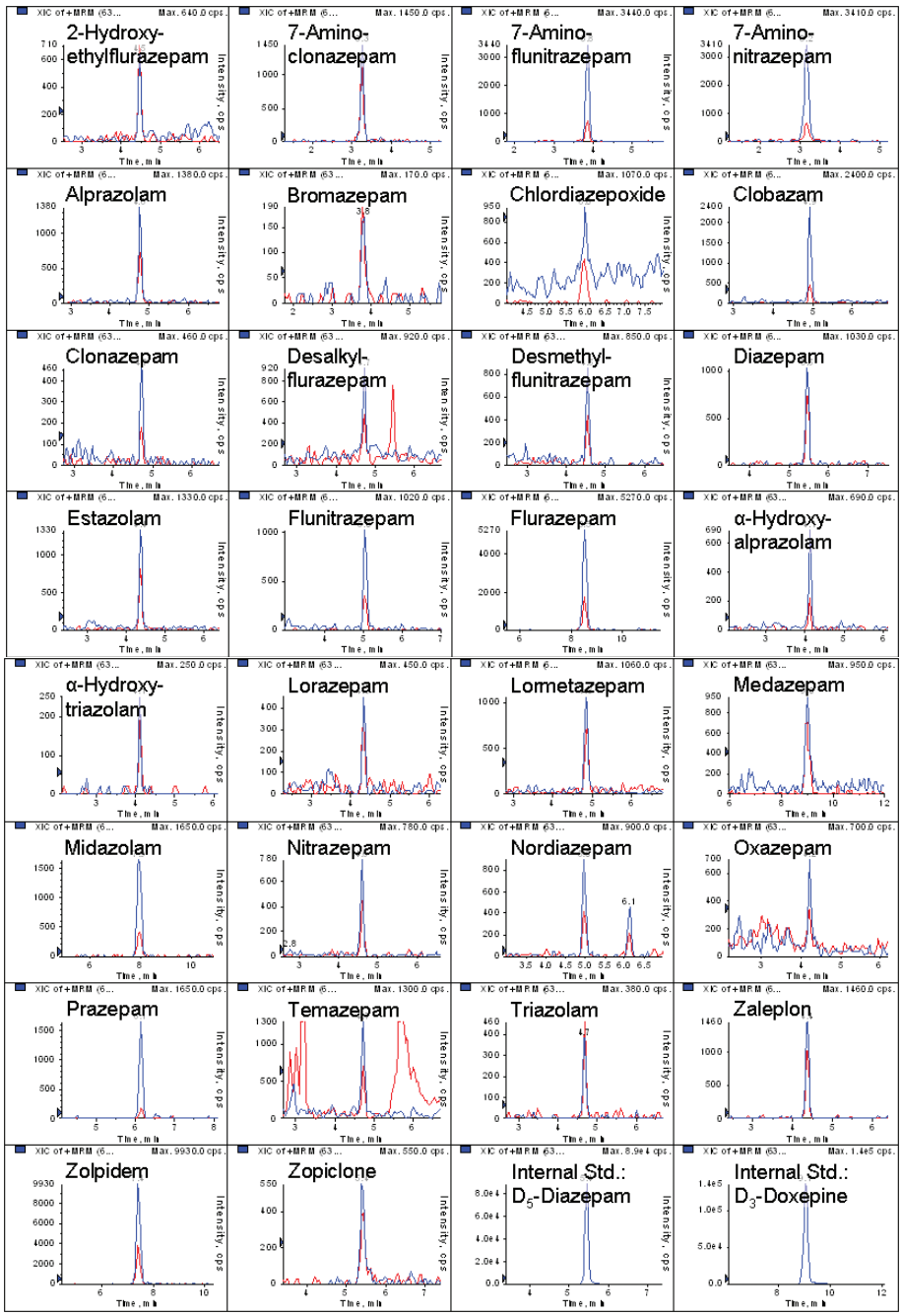


Figure 2. High sensitivity and selectivity of LC/MS/MS detection of 10ng/mL of Benzodiazepines and nonbenzodiazepine hypnotics in urine samples after sample preparation (blue trace: quantifier MRM and red trace: qualifier MRM).

Automatic Data Processing

The developed method for the quantitation and confirmation of benzodiazepines and nonbenzodiazepine hypnotics is built into Cliquid™ Drug Screen & Quant Software.[4] Preconfigured report styles allow an automatic reporting regarding forensic guidelines. An example report of a forensic urine sample tested positive for Diazepam (1.6ng/mL), Nordiazepam (670ng/mL), and Temazepam (470ng/mL) is shown in Figure 5. The yellow highlighting in the results table indicates a positive confirmation based on the detected MRM ratio.

Summary

The developed LC/MS/MS method for the quantitation and confirmation of 27 benzodiazepines and 3 nonbenzodiazepine hypnotics was successfully applied for the analysis of forensic case urine samples. The targeted compounds had Limits of Quantitation between 0.1 and 10ng/mL in urine after simple and fast sample preparation which consists of the addition of internal standards and 1/10 dilution. The dilution step was proven to be sufficient to minimize matrix effects during ionization to allow an accurate and reproducible quantitation.

The developed LC/MS/MS method is preconfigured in Cliquid™ Drug Screen & Quant Software to allow testing in routine forensic toxicology or clinical research with automatic reporting of quantitative results and MRM ratio confirmation.

Future developments will include the detection of glucuronide metabolites of benzodiazepines by either extended sample preparation with hydrolysis or the direct detection of MRM transitions of respective metabolites.

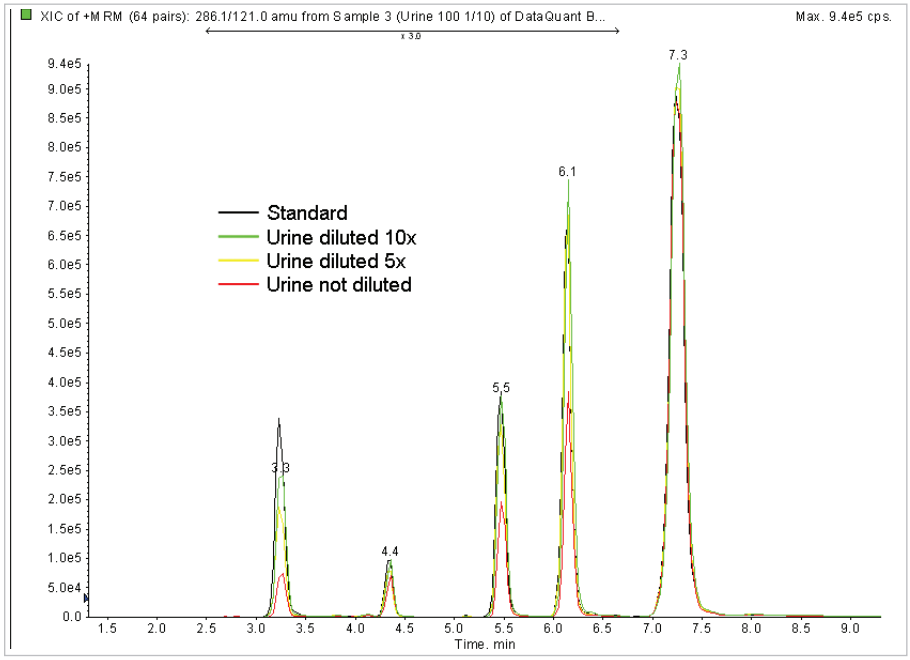


Figure 3. Minimizing ion suppression of 7-Aminoclonazepam (3.3min), Lorazepam (4.4min), Diazepam (5.5min), Prazepam (6.1min), and Zolpidem (7.3min) by dilution of urine.

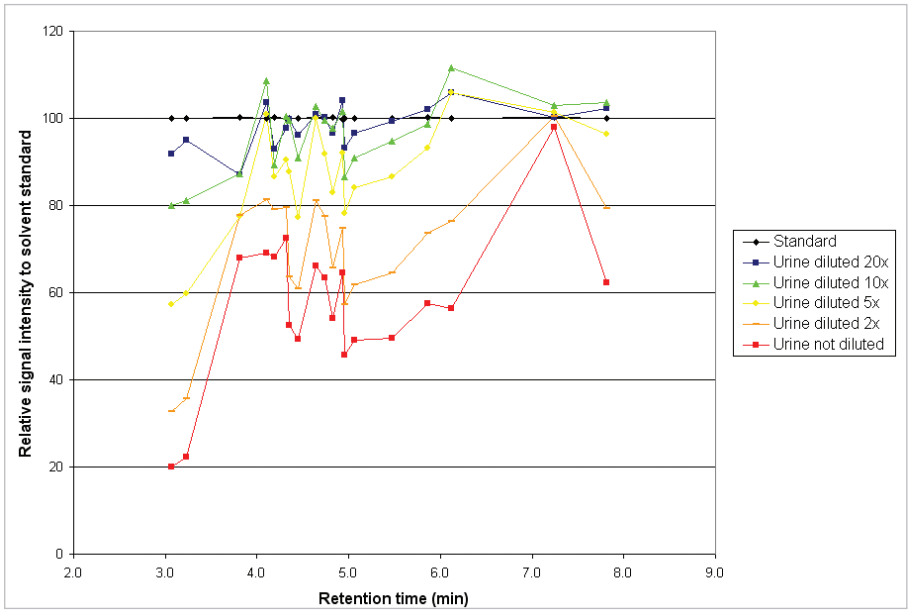
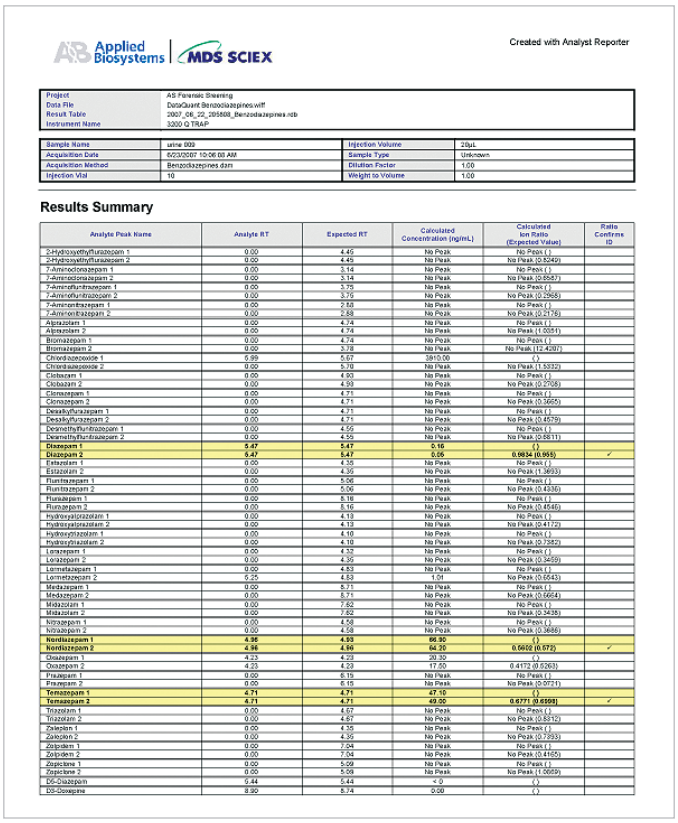


Figure 4. Ion suppression effects depending on retention time and dilution factor of urine sample prior to LC/MS/MS analysis using Electrospray Ionization (ESI).

TABLE 2. MULTIPLE REACTION MONITORING (MRM) TRANSITIONS, COMPOUND DEPENDANT PARAMETERS, AND RETENTION TIMES, LIMIT OF QUANTITATION (LOQ) IN URINE BASED ON THE QUALIFIER MRM TRANSITION, AND COEFFICIENT OF VARIATION (n=3) OF ALL DETECTED SUBSTANCES

Substance	Q1 (amu)	Q3 (amu) quantifier	Q 3 (amu) qualifier	DP (V)	CE (V) quantifier	CE (V) qualifier	Retention time (min)	LOQ ng/mL (S/N=6)	% CV (10ng/mL)
2-Hydroxyethylflurazepam	333.1	211.2	109.0	56	51	41	4.5	1	6.3
7-Aminoclonazepam	286.1	121.0	222.2	46	41	35	3.3	0.5	0.6
7-Aminoflunitrazepam	284.1	135.1	226.0	51	39	49	3.8	0.5	1.6
7-Aminonitrazepam	252.1	121.1	94.0	51	35	53	3.2	1	4.3
Alprazolam	309.1	205.1	281.1	56	53	35	4.8	1	3.1
Bromazepam	316.0/318.0	182.1	182.1	51	45	45	3.8	5	7.7
Chlordiazepoxide	300.1	227.1	283.2	36	31	21	6.0	5	3.8
Clobazam	301.1	259.1	224.3	46	29	47	4.9	1	2.9
Clonazepam	316.0	270.2	214.0	56	41	51	4.7	2	8.3
Desalkylflurazepam	289.1	140.1	226.1	71	41	39	4.7	2	8.5
Desmethylflunitrazepam	300.1	254.2	198.2	56	35	51	4.5	2	7.9
Diazepam	285.0	193.2	154.1	55	41	37	5.5	1	10.1
Estazolam	295.0	205.0	267.1	51	53	31	4.4	2	7.0
Flunitrazepam	314.0	268.1	239.1	56	35	49	5.0	1	4.3
Flurazepam	388.2	315.1	317.1	36	27	27	8.5	0.1	3.1
α-Hydroxyalprazolam	325.1	297.2	204.9	51	31	59	4.1	2	7.3
α-Hydroxytriazolam	359.0	239.2	176.0	61	63	37	4.1	5	6.5
Lorazepam	321.0/323.1	275.0	277.0	41	31	27	4.3	5	7.9
Lormetazepam	335.0/337.1	289.0	291.1	41	29	29	4.8	2	6.4
Medazepam	271.0	91.1	207.3	46	41	39	9.0	2	5.4
Midazolam	326.1	291.3	222.0	56	33	63	7.9	0.5	6.0
Nitrazepam	282.0	236.1	180.2	71	35	51	4.6	2	7.7
Nordiazepam	271.1	140.2	164.9	46	37	35	5.0	2	6.7
Oxazepam	287.0	241.1	268.9	41	27	19	4.2	10	7.6
Prazepam	325.1	271.1	140.0	81	31	53	6.1	2	6.6
Temazepam	301.1/303.1	255.1	257.2	35	30	30	4.7	5	3.9
Triazolam	343.0	238.9	314.9	61	53	37	4.7	1	1.7
Zaleplon	306.2	236.3	264.2	56	35	27	4.4	0.5	10.2
Zolpidem	308.2	235.1	236.1	56	39	35	7.4	0.2	1.8
Zopiclone	389.1	244.8	217.0	16	25	41	5.4	1	5.3
D_5-Diazepam	290.1	198.2	-	55	41	-	5.4	-	-
D_3-Doxepine	283.0	107.1	-	41	35	-	9.1	-	-



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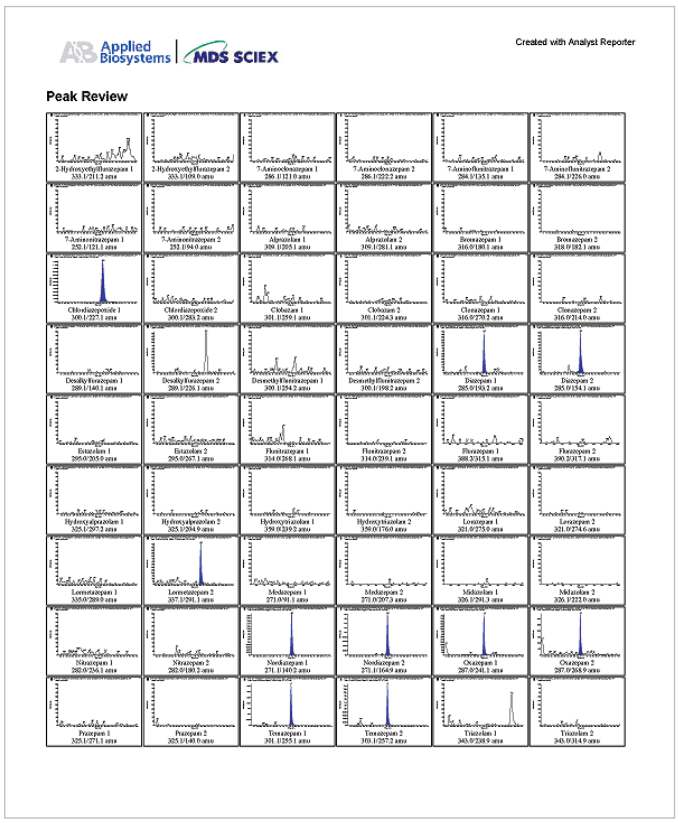
Project	AS Forensic Screening
Data File	DataQuant Benzodiazepines.wiff
Result Table	2007_06_22_205800_Benzodiazepines.rdb
Instrument Name	3200 Q TRAP

Sample Name	urine 009	Injection Volume	20µL
Acquisition Date	6/23/2007 10:06:00 AM	Sample Type	Unknown
Acquisition Method	Benzodiazepines.dam	Dilution Factor	1.00
Injection Vial	10	Weight to Volume	1.00

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calculated Concentration (ng/mL)	Calculated Ion Ratio (Expected Value)	Ratio Confirms ID
2-Hydroxyethylflurazepam 1	0.00	4.45	No Peak	No Peak ( )	
2-Hydroxyethylflurazepam 2	0.00	4.45	No Peak	No Peak (0.8249)	
7-Aminoclonazepam 1	0.00	3.14	No Peak	No Peak ( )	
7-Aminoclonazepam 2	0.00	3.14	No Peak	No Peak (0.8587)	
7-Aminoflunitrazepam 1	0.00	3.75	No Peak	No Peak ( )	
7-Aminoflunitrazepam 2	0.00	3.79	No Peak	No Peak (0.2968)	
7-Aminonitrazepam 1	0.00	2.58	No Peak	No Peak ( )	
7-Aminonitrazepam 2	0.00	2.88	No Peak	No Peak (0.2178)	
Alprazolam 1	0.00	4.74	No Peak	No Peak ( )	
Alprazolam 2	0.00	4.74	No Peak	No Peak (1.0251)	
Bromazepam 1	0.00	4.74	No Peak	No Peak ( )	
Bromazepam 2	0.00	3.78	No Peak	No Peak (12.4287)	
Chlordiazepoxide 1	5.99	5.67	3910.00	( )	
Chlordiazepoxide 2	0.00	5.70	No Peak	No Peak (1.5332)	
Clobazam 1	0.00	4.93	No Peak	No Peak ( )	
Clobazam 2	0.00	4.93	No Peak	No Peak (0.2708)	
Clonazepam 1	0.00	4.71	No Peak	No Peak ( )	
Clonazepam 2	0.00	4.71	No Peak	No Peak (0.3665)	
Desalkylflurazepam 1	0.00	4.71	No Peak	No Peak ( )	
Desalkylflurazepam 2	0.00	4.71	No Peak	No Peak (0.4579)	
Desmethylflunitrazepam 1	0.00	4.56	No Peak	No Peak ( )	
Desmethylflunitrazepam 2	0.00	4.59	No Peak	No Peak (0.5811)	
Diazepam 1	**5.47**	**5.47**	**0.16**	**( )**	
Diazepam 2	**5.47**	**5.47**	**0.05**	**0.0834 (0.055)**	✓
Estazolam 1	0.00	4.35	No Peak	No Peak ( )	
Estazolam 2	0.00	4.35	No Peak	No Peak (1.3693)	
Flunitrazepam 1	0.00	5.06	No Peak	No Peak ( )	
Flunitrazepam 2	0.00	5.06	No Peak	No Peak (0.4336)	
Flurazepam 1	0.00	5.16	No Peak	No Peak ( )	
Flurazepam 2	0.00	5.16	No Peak	No Peak (0.4546)	
Hydroxyalprazolam 1	0.00	4.13	No Peak	No Peak ( )	
Hydroxyalprazolam 2	0.00	4.13	No Peak	No Peak (0.4172)	
Hydroxytriazolam 1	0.00	4.10	No Peak	No Peak ( )	
Hydroxytriazolam 2	0.00	4.10	No Peak	No Peak (0.7352)	
Lorazepam 1	0.00	4.32	No Peak	No Peak ( )	
Lorazepam 2	0.00	4.35	No Peak	No Peak (0.3459)	
Lormetazepam 1	0.00	4.53	No Peak	No Peak ( )	
Lormetazepam 2	5.25	4.53	1.01	No Peak (0.6543)	
Medazepam 1	0.00	8.71	No Peak	No Peak ( )	
Medazepam 2	0.00	8.71	No Peak	No Peak (0.6664)	
Midazolam 1	0.00	7.82	No Peak	No Peak ( )	
Midazolam 2	0.00	7.82	No Peak	No Peak (0.3438)	
Nitrazepam 1	0.00	4.58	No Peak	No Peak ( )	
Nitrazepam 2	0.00	4.58	No Peak	No Peak (0.3886)	
Nordiazepam 1	**4.96**	**4.93**	**66.80**	**( )**	
Nordiazepam 2	**4.96**	**4.96**	**64.20**	**0.0602 (0.072)**	✓
Oxazepam 1	4.23	4.23	20.30	( )	
Oxazepam 2	4.23	4.23	17.50	0.4172 (0.5263)	
Prazepam 1	0.00	6.15	No Peak	No Peak ( )	
Prazepam 2	0.00	6.15	No Peak	No Peak (0.07721)	
Temazepam 1	**4.71**	**4.71**	**47.10**	**( )**	
Temazepam 2	**4.71**	**4.71**	**49.00**	**0.6771 (0.6698)**	✓
Triazolam 1	0.00	4.67	No Peak	No Peak ( )	
Triazolam 2	0.00	4.67	No Peak	No Peak (0.8312)	
Zaleplon 1	0.00	4.35	No Peak	No Peak ( )	
Zaleplon 2	0.00	4.35	No Peak	No Peak (0.7293)	
Zolpidem 1	0.00	7.04	No Peak	No Peak ( )	
Zolpidem 2	0.00	7.04	No Peak	No Peak (0.4165)	
Zopiclone 1	0.00	5.09	No Peak	No Peak ( )	
Zopiclone 2	0.00	5.09	No Peak	No Peak (1.0669)	
D5-Diazepam	5.44	5.44	< 0	( )	
D3-Doxepine	8.90	8.74	0.00	( )	



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Peak Review

Figure 5. Example report generated automatically by Cliquid™ Drug Screen & Quant Software of a urine sample tested positive for benzodiazepines (1.6ng/mL Diazepam, 670ng/mL Nordiazepam, and 470ng/mL Temazepam).

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